Arizona Iceberg Lettuce Research Council
Project Annual Report: July 2003 – September 2004

Project Title: Detection of *Fusarium oxysporum* f. sp. *lactucae* in lettuce seed and soil

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Purpose of the Study

In 2001, a serious lettuce disease, Fusarium wilt, appeared in 5 fields near Wellton, AZ. Although this disease had been previously reported in California and in Japan, this was the first report in Arizona. In 2002, the pathogen was reported in additional fields in the Gila Valley and near Yuma, and new reports have continued in 2003 and 2004. It is apparent that the pathogen is spreading in the winter lettuce growing regions of Arizona. As a relatively new lettuce disease, very little is known about it beyond the fact it is caused by *Fusarium oxysporum* f. sp. *lactucae*. In addition, few options are available to growers to combat this disease and prevent potentially significant yield loss. Of paramount importance in the study of any plant pathogen is the ability to detect the organism at low population densities and from various substrates. Modern molecular techniques permit the ability to detect organism by the presence of their DNA. These techniques have the additional advantage of being highly specific in their detection and incredibly sensitive. For this project, molecular techniques are employed to detect the fungus *F. oxysporum* f.sp. *lactucae* in lettuce seed, tissue, and field soil. The project was initiated by determining the genetic relationship of this fungus to other strains of *Fusarium oxysporum* and to other fungi using DNA sequence analysis of five different genomic regions. Only one of these regions, the rDNA IGS region, was variable enough to resolve the *lactucae* isolates into a group distinct from other *Fusarium* species and other fungi. Based on this unique DNA region, we have developed a molecular method to detect the fungus in both seed and soil. In addition, studies have been initiated to monitor the movement of specific genotypes (populations) in the lettuce growing areas around Yuma, and to use this information to deduce the origins of the pathogen in Arizona.

Project Objectives:

- Develop DNA-based PCR primers to discriminate between lettuce-pathogenic *Fusarium* and other related species
- Develop a PCR-based seed assay to detect the pathogen in lettuce seed
- Develop a PCR-based soil assay to detect the pathogen in field soil (and also in lettuce tissue)
- Determine if *Fusarium* contamination of seed can occur in greenhouse studies
- Screen commercial lettuce seed lots for the presence of the lettuce pathogen
- Examine population genetic diversity of the pathogen to understand movement within Arizona and between California and Arizona

Results:

1. We have found regions of DNA in the *Fusarium* genome unique to isolates causing *Fusarium* wilt of lettuce. Using these sequences, we have developed a DNA-based detection method using PCR techniques that will detect only DNA from lettuce-pathogenic isolates of *Fusarium*, even in the presence of much larger amounts of non-pathogenic *Fusarium* DNA. However, the initial PCR primers developed for this method produced considerable background noise, and could amplify genomic DNA at a concentration of only 20 picograms (10^{-12} grams). The sensitivity of the method was improved with the design of a modified primer that differed by one base at the 3' end of the original primer. Using this modified primer, genomic DNA concentrations as low as 2 femtograms (10^{-15} grams) could be detected. (Figure 1). In specificity tests, DNA was amplified from the *lactucae* isolates and not from other *Fusarium oxysporum* isolates or fungal species (Figure 2).

2. The PCR method developed was able to detect pathogenic *Fusarium* DNA in extracts of lettuce seed that have been artificially inoculated with target DNA. In these experiments, the DNA was amplified from seed lots with infestation rates as low as 0.1% (Figure 3). At very low infestation rates, detection of the pathogen was not always consistent in duplicate samples due to the statistical probability of each seed lot subsample containing an infested seed. Thus, it is critical to establish a statistically robust sampling protocol and conduct analysis in replications to insure consistent detection at low levels of infestation. These protocols and procedures have been established for other pathogens (e.g., lettuce mosaic virus) and should be directly applicable for *Fusarium* detection. A manuscript is in preparation that describes this research in detail and the resulting PCR-based seed assay method. We believe this method is now ready for use by all parties interested in detecting *F. oxysporum* f.sp. *lactucae* in lettuce seed.

3. In our most recent soil assay experiments, soil samples of two infested fields from Wellton were assayed for *lactucae* using our PCR-based detection method. First, total DNA was extracted from the soil samples. Serial dilutions were made with pure fungal DNA from one of the *lactucae* isolates and then added to the soil-extracted DNA. Using a modification of the PCR-based seed assay, the lactucae DNA was amplified from three of the six soil extracts that contained respectively 10^{-1}, 10^{-2}, and 10^{-5} ng/µl of fungal DNA, but not from the soil extracts that contained no added fungus DNA (Figure 4). These results reveal that the PCR protocol can successfully detect *lactucae* DNA from spiked soil DNA extracts. However, amplification is not as sensitive as with seed assays, and amplification is not consistent. The primary problem with DNA detection in soil extracts is the presence of humic and phenolic substances in the extracts that impede the detection of DNA except at very high levels. Currently, efforts are underway to improve extraction procedures and reduce or eliminate all humic and phenolic compounds during DNA purification from soil. When the sensitivity of the soil detection protocol has been optimized, *Fusarium* distribution/density in soil will be evaluated. These data will be used to develop statistically sound sampling methods that will improve the chances of pathogen detection in naturally infested soil at varying population densities.

4. Studies were performed in the greenhouse to determine if lettuce seed could become naturally infected by *F. oxysporum* f.sp. *lactucae* following infection of the lettuce plant. Both crisphead and leaf cultivars were used in these experiments. Plants were inoculated with the pathogen at the roots using two different inoculation methods, and the movement of the pathogen up the flower stalk and into the seed was monitored. When the pathogen was introduced to lettuce roots during transplanting, it could usually be recovered at all points up the flower stalk and into the flower pedicel at lettuce maturity, but usually not from the seed (Table 1). However, in 2% of the inoculations the pathogen was recovered from the seed as well, confirming infected that seed can be produced from infected plants in some cases. Interestingly, the detection of the pathogen in the seed was not possible using standard agar medium plating techniques, and only could be detected using our DNA-based detection method. In addition to pathogen movement up the lettuce plant, when uninfected seed was mixed with infected plant material and then cleaned, the resulting seed was usually infested, confirming that seed can easily become infested during the threshing process.

5. 25 commercial lots were obtained from commercial seed companies in 2003-2004 and were examined for the presence of *lactucae* by both agar plating methods and by our DNA-based seed assay method. Each assay examined 1 gram of seed (approx. 800-1000 seeds) at a time. For each lot, 5 grams of seed were examined (5 replications = approx. 5000 seeds). To date, 4 of the 25 commercial lots tested positive for the presence of *lactucae* (Table 2). These positive results were obtained only by the DNA-based method, and not by the agar plating method. It must be stressed that these are preliminary results, and all tests, positive and negative, are currently being repeated to confirm findings. In addition, any suspect *lactucae* isolates recovered from these lots will be tested for pathogenicity on susceptible lettuce cultivars. This is a critical confirmation step to insure that these results are not false positives and that each lot actually contained pathogenic *F. oxysporum* f.sp. *lactucae.*

6. Population studies were conducted to characterize variation among *lactucae* isolates and changes in populations in AZ over time. These studies were based upon DNA fingerprint analysis, rather than DNA sequence analysis used in the systematic study. Fingerprint analysis can differentiate individual genotypes of *lactucae* and this information can be used to establish the distribution of these genotypes across specific locations. Most of the AZ isolates recovered in 2002 were identical and matched exactly the HL1 and HL2 isolates from the original CA infestation in 1990 (Figure 5). Interestingly, these also matched the Italy isolates recovered in 2002. A few AZ isolates recovered in 2003 and 2004 were new genotypes and could represent new introductions the fungus or an evolution of the genotypes already found in Arizona (Figure 6). California isolates are much more diverse than Arizona isolates, which is consistent with the fact that the pathogen has reported in California for a considerable longer period of time. It is interesting to note that the diversity in California encompasses the diversity found in all other locations reported; Japan, Taiwan, Italy, and Arizona. Isolates from Iran, where the pathogen has also been reported, were not available for analysis.

Future Research Efforts

The finding of possible *Fusarium*-positive seed lots is serious and must be confirmed with absolute certainty. Although we are quite confident in our methods, all tests are being repeated with special attention to preventing any possible cross-contamination. There is also some possibility that cross-reactions are occurring with some unknown and untested seed-borne fungus. Therefore, the recovery of viable *F. oxysporum* f.sp. *lactucae* isolates from each positive seed lot, and confirmation of pathogenicity in greenhouse tests is a critical step that must be made before discussion of seed-borne inoculum can proceed any further. Results of these test should be available by December 2004. In addition, more commercial seed lots will be obtained and assayed to confirm or refute findings of 2003-2004 research. Furthermore, the use of real-time PCR will be investigated to see if this method can be used to determine the percent infestation of seed lots in addition to simply the presence or absence of the pathogen.

The method developed for assaying seeds for the presence of the pathogen also works for soil assays. However, the present challenge is to improve the sensitivity of the soil assay such that it can detect the fungus in soil at very low soil population densities. The limit of detection will be determined using soil from heavily infested fields after soil plate methods have conclusively determined the presence of the pathogen. All fields from which the pathogen has been documented to-date will be subsequently sampled and soil dilutions will be tested to generate population data based upon densities. Following the determination of natural range of population densities, subsequent studies will examine the spatial distribution of the pathogen across fields.

Research will continue to evaluate population variation among isolates of *Fusarium* from newly infested fields in order to genotype any new introductions or changing strains. In addition, the movement of specific genotypes into new fields or lettuce-growing regions will be monitored. Additional strains from California will be analyzed to confirm the broad diversity found in the state and relate this diversity to that found in other regions and countries.

Table 1: Recovery of *Fusarium oxysporum* f. sp. *lactucae* from lettuce plants at maturity following inoculation during transplanting. Recovery was based upon a standard agar plating technique on Komada's medium. The pathogen was not detected on seed using this recovery technique. However, the pathogen was detected on a small number of seed samples using the DNA-based detection method.

Cultivar	Type of Inoculation	Plant Parts					
		Basal nodes	Middle nodes	Upper nodes	Flower pedicel	Seed (by agar)	Seed (by PCR)
Sharpshooter	Root dip	+	+	+	+	-	-
	Soil inoc.	+	+	+	+	-	+
King Louie	Root dip	+	+	+	+	-	-
	Soil inoc.	+	+	+	+	-	-
Vulcan	Root dip	+	+	+	+	-	-
	Soil inoc.	+	+	+	+	-	+

Figure 1. Amplification of serially diluted DNA from *F. oxysporum* f.sp. *lactucae*. **A.** improved sensitivity with new primer set. **B.** Level of sensitivity with originally designed primer set (note the background noise and limit of detection).

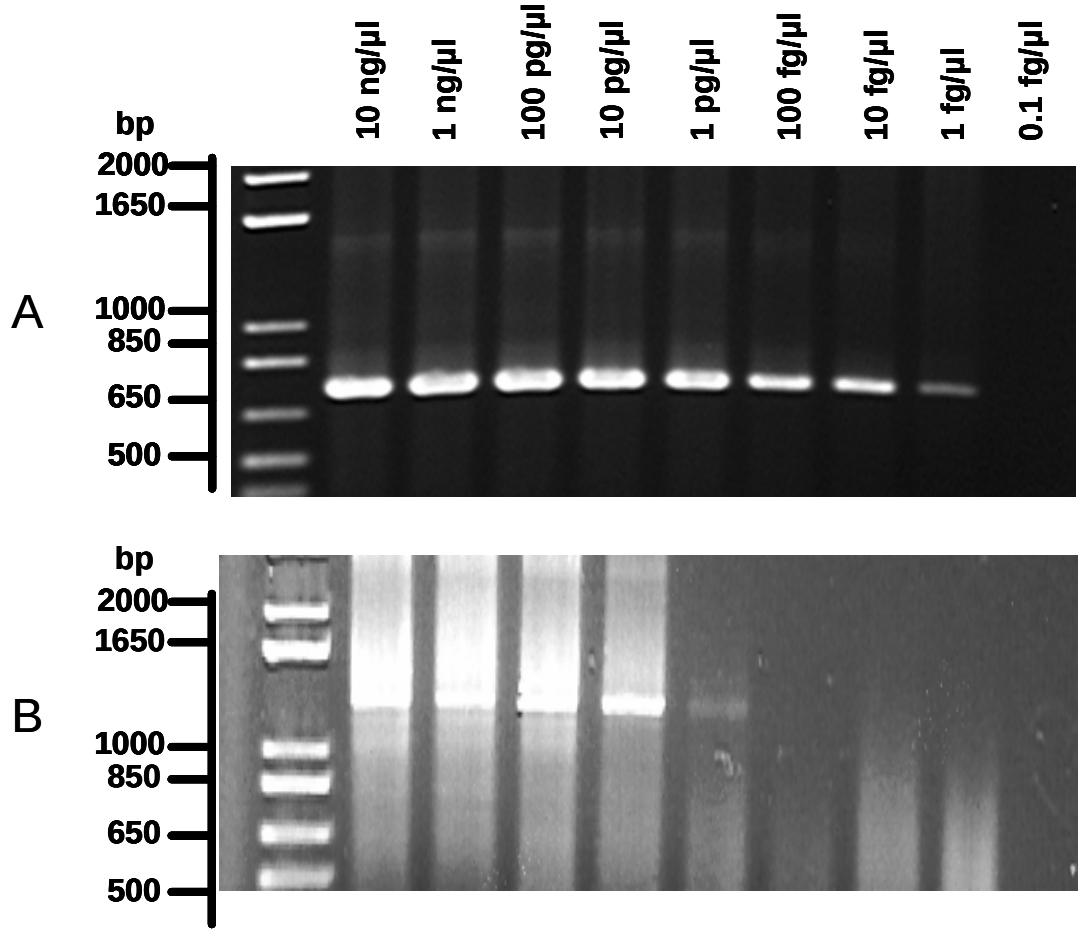

Figure 2. Specificity of primers: amplification of DNA from *lactucae* isolates but not other *Fusarium* isolates and species.

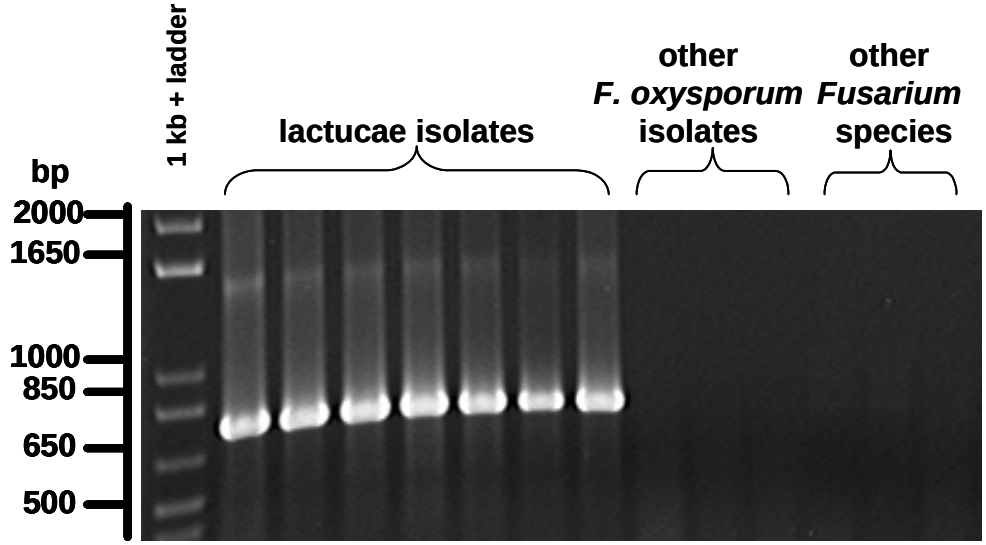

Figure 3 Limit of detection in seeds with different levels of artificial infestations. Target could be detected in seed with infestation level as low as 0.1%. Results display detection in duplicate analyses.

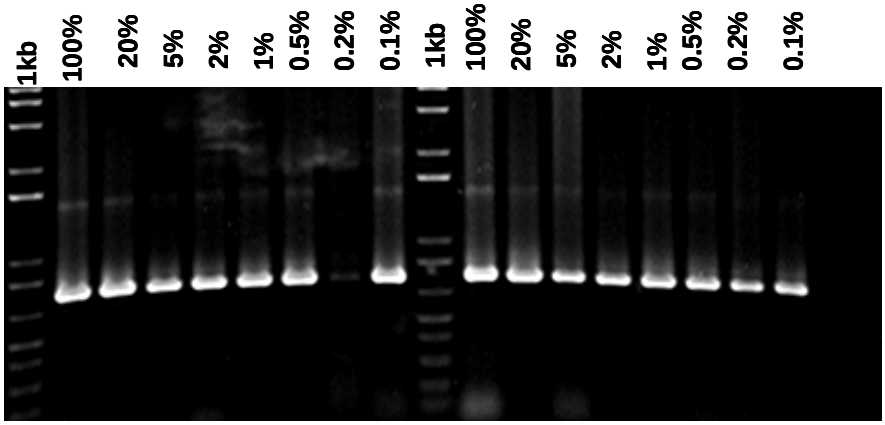

Figure 4. Soil assay using primers specific for *Fusarium oxysporum* pathogenic on lettuce. Soil sample obtained from an infested field in Wellton. Six independent DNA isolations were done on 1g each of soil sample. The samples were bulked and spiked with 2 µl of serially diluted genomic DNA from a pathogenic isolate. The lanes are as follows: 1 - unspiked soil DNA extract, 2-7 - soil DNA extract spiked with decreasing amounts of pure *lactucae* DNA, and 8 - pure *lactucae* DNA.

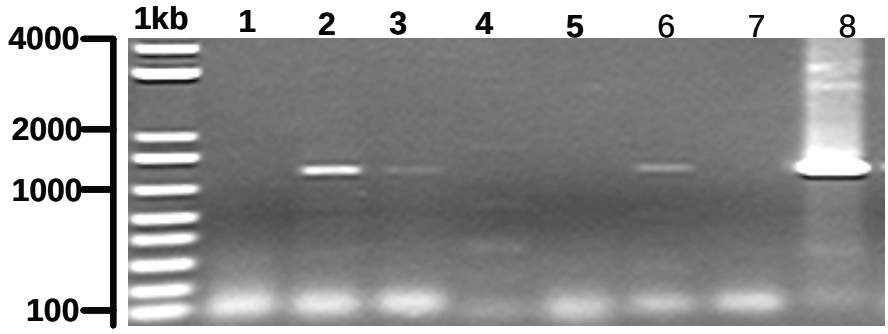

Figure 5: Genetic diversity of *F. o. lactucae* based upon DNA fingerprint analysis (microsatellite analysis). This phenogram was genetic from a combined fingerprint data of five microsatellite primers. Origin and race of the isolates are given. NP represents non-pathogenic soil isolates.

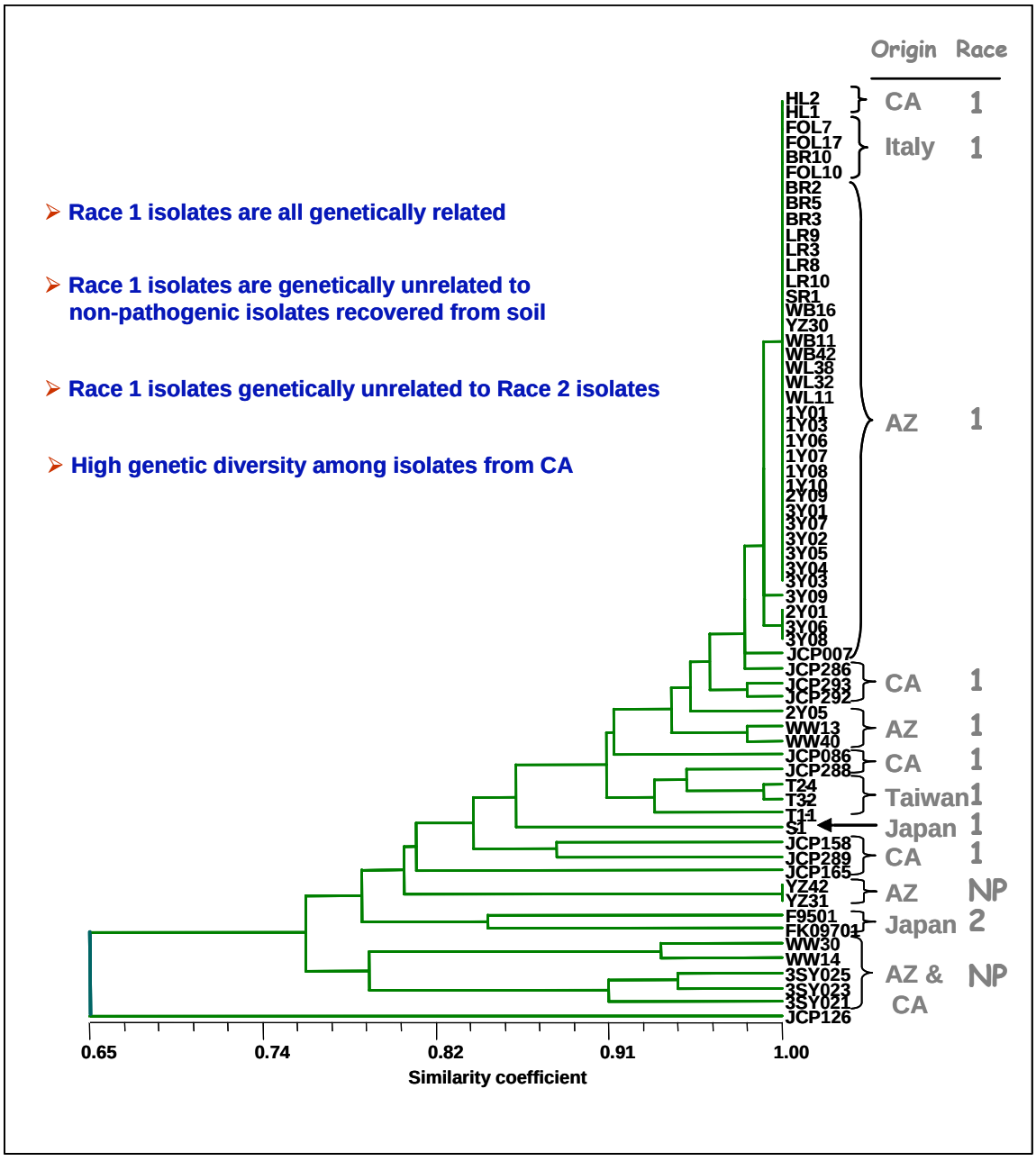

Figure 6: Distribution of genotypes in lettuce fields in AZ 2002-2004. Genotypes designated by colored ovals: white, blue, yellow, red, grey, green. Position of oval represents location of field where genotype was recovered. Note: Stars represent location of infested fields still requiring pathogen isolation and genotyping.